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(54) **METHOD OF ISOLATING CA 125 ANTIGEN**

VERFAHREN ZUR ISOLIERUNG VON CA 125-ANTIGEN

PROCEDE D'ISOLATION DE L'ANTIGENE CA 125

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- Chemical Abstracts, vol. 107, no.19, 9 November 1987 (Columbus, Ohio, US) J.A. Holt et al "Serum CA 125 and survival of mice inoculated with ovarian carcinoma and treated with antilestrogen, estrogen or progestin", see page 81, abstract 1569012u
- Chemical Abstracts, vol. 108, no.3, 18 January 1988 (Columbus, Ohio, US) F.Leoni et al "Immunochemical characterization and radioimmunochemical detection of molecules shed by human ovarian cancer", see abstract 20206y
- Gel filtration, Principles and Methods, p. 17, 5th ed., Serial no. 18-1022-18, Pharmacia LKB Biotechnology, Uppsala, Sweden.
- Krupey, J., Gold P. and Freedman, s.o., 1968, J. of Experimental Medicine, 128, pp.387-398.

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DescriptionBackground

CA 125 is a tumor-associated antigen expressed on greater than 80% of all non-mucinous ovarian epithelial tumors of serous, endometrioid, clear cell and undifferentiated histologies. Bast, R.C., Jr. *et. al.*, *J. Clin. Invest.* **68**:1331-1337, 1981; Kabawat, S.E. *et. al.*, *Am. J. Clin. Pathol.*, **79**:98-104, 1983. The murine monoclonal antibody OC125 which reacts with CA 125 was generated by use of an established human serous cystadenocarcinoma cell line, OVCA 433. Bast, R.C., Jr. *et. al.*, *supra*. Quantitation of this determinant in serum of patients with ovarian cancer has been made possible by the development of an immunoradiometric assay with OC125. Klug, T.L. *et. al.*, *Cancer Res.*, **44**:1048-1053, 1984. The CA 125 antigenic determinant has also been reported to be found in human milk (Hanisch, F.G. *et. al.*, *Eur. J. Biochem.*, **149**:323-330, 1985.), in normal cervical mucous (de Bruijn, H.W.A. *et. al.*, *Am. J. Obstet. Gynecol.*, in press) and in the central airway and normal lung tissue (Nouwen, E.J. *et. al.*, *Cancer Res.*, **43**:866-876, 1986). In addition, CA 125 activity appears to exist in human seminal plasma.

The CA 125 determinant has been reported to be associated with a mucin-like high molecular weight glycoprotein complex. See e.g., Hanisch, F.G. *et. al.*, *Eur. J. Biochem.*, **149**:323-330, 1985; Niloff, J.M. *et. al.*, *Am. J. Obstet. Gynecol.*, **151**:981-986, 1986; Bast, R.C. *et. al.*, *Ovarian Cancer*, pp. 23-35, Boston, MA: Martinus Nihoff, 1985; Masuho, Y. *et. al.*, *Cancer Res.*, **44**:2813-2819, 1984; and Bast, R.C., Jr. *et. al.*, *Cancer Bull.*, **37**:80-81, 1985. However, the lack of procedure for isolation of CA 125 antigen has impaired analysis of its chemical composition.

Disclosure of the Invention

This invention pertains to a method of isolating the antigen CA 125, to preparations of the isolated antigen and to methods of using the isolated antigen.

The CA 125 antigen can be isolated in high purity as a 200 kDa species from tissue culture medium of ovarian carcinoma cells which "shed" the antigen into the growth medium, (e.g. the human serous cystadenocarcinoma cell line OVCA 433). The CA 125 species isolated by the procedure described herein is the same as the species or CA 125 found in the serum of patients with non-mucinous ovarian carcinoma; as determined by electrophoretic and immunoblotting analysis.

According to the procedure of this invention, the cell free supernatant is obtained from a culture of human ovarian carcinoma cells. In a first step, protein is precipitated by acid treatment (e.g. perchloric acid, 6M) and the precipitated protein is removed. The acid soluble fraction which contains the CA 125 activity is then neutralized.

CA 125 activity in the acid soluble fraction is associated with a high molecular weight complex (1,000,000 Da). In the next step, molecular size exclusion chromatography is then used to separate this high molecular weight CA 125 species from lower molecular weight components. For example, the acid soluble fraction can be applied to a column of Sepharose™ 4B-CL gel. The Sepharose™ 4B-CL retains molecules of about 60,000-2,000,000 Da. The CA 125 complex is eluted from this column in the void volume.

A chaotropic agent (e.g. Urea, 6M) is used to disrupt the high molecular weight CA 125 complex separated by molecular size exclusion chromatography. The chaotropic agent can be added to the CA 125-containing fraction from the Sepharose™ 4B-CL column. The CA 125 is then separated by a second molecular size exclusion chromatography step. This column is chosen to retain a 200, kDa CA 125 species (e.g. Sepharose™ 6B resin). The chromatography is performed with an elution buffer containing the chaotropic agent and a detergent (e.g. SDS) to stabilize the disrupted CA 125. The retained fractions containing CA 125 activity (as determined reactivity with OC125 antibody) are collected as they are eluted from the column. The chaotropic agent is removed from the collected fraction e.g. by dialysis.

In the final step of the isolation procedure, the CA 125 is immunopurified using OC125 antibody. For this purpose, an immunoaffinity column comprising immunoactive OC125 coupled to a resin material (e.g. Sepharose 4B) is used.

The CA 125 antigen isolated by this procedure has a molecular weight of about 200kDa and a bouyant density of about 1.36 g/ml. The antigen is 24% carbohydrate (by mass). Antibody binding (OC125) activity is heat and protease labile but exoglycosidase and periodate insensitive, indicating that the cognate determinant of OC125 is likely to be proteinaceous.

Isolated CA 125 antigen can be used to raise polyclonal or monoclonal antibody reactive with CA 125. Antibody against OC125 can be used in diagnosis and/or therapy of ovarian carcinoma. e.g. tumor imaging, passive immunotherapy, and immunotoxin therapy. Further, the isolated CA 125 antigen can be used for detection of anti-CA 125 antibody (e.g. by solid phase RIA or ELISA) in the serum, plasma or other biological fluids of patients. The presence of anti-CA 125 antibody in a patient may provide an indication of the existence or recurrence of ovarian carcinoma.

Brief Description of the Drawings



Figure 1 is the elution profile on Sepharose CL-4B column chromatography of the CA 125 antigen isolated from OVCA 433 tissue culture supernatant () and from human serum ().






Figure 2 shows density gradient ultra-centrifugation following Sepharose CL-4b column chromatography of the CA 125 antigen isolated from human serum (), OVCA 433 tissue culture supernatant (), and from human milk ().

Figure 3 shows SDS-PAGE of CA 125 antigen isolated from human milk/4B (200 units/lane) (lane 1), OVCA 433 passage 69/4B (300 units/lane) (lane 3), human ovarian cancer patient sera/4B (100-200 units/lane) (lane 4-7), and from a negative control serum/4B (23 units/lane) (lane 8).

Figure 4 shows conventional SDS-PAGE (3-12% gradient) of CA 125 antigen isolated from OVCA 433 followed by immunoblotting.

Figure 5A shows Sepharose CL-6B elution profile of the CA 125 antigen isolated from OVCA 433. Elution was performed in a SDS-urea-Tris buffer following treatment in 6 M urea at 45°C for 30 min. Fractions were assayed for CA 125 activity with a solid-phase RIA. Figure 5B shows the SDS-PAGE (6%) of pertinent fractions of the Sepharose CL-6B gel filtration column chromatography.

Figure 6 shows density gradient ultra-centrifugation of the CA 125 antigen isolated from OVCA 433 following partial purification on a Sepharose CL-4B column () and following immunoaffinity chromatography purification on an immobilized OC125-Protein A-Sepharose CL-4B column ().

Detailed Description of the Invention

The procedure for purifying CA 125 generally entails four steps, as indicated below. The procedure can be applied to isolate CA 125 from tissue culture media in which ovarian carcinoma cells have been grown. The cells, of course, must be ovarian carcinoma cells which express the antigen and "shed" (release) the antigen into the growth medium. The procedure may also be used to isolate the antigen from biological fluids such as serum or ascites. However, the minute quantities found in these fluids generally make them an impracticable source of the antigen for purification.

The preferred ovarian carcinoma cell line is the OVCA 433 cell line described by Bast, R.J., Jr. *et al.*, *supra*. Other cell lines which can be used are the ovarian tumor cell lines NIH:OVCA-3 (ATCC #HTB161), SK-OV-3 (ATCC # HTB77), CAOV-3 (ATCC # HTB75), and CAOV-4 (ATCC # HTB76). When grown in a conventional tissue medium, these cell lines release CA 125 antigen into the medium. The released antigen can then be isolated from the medium by the four step procedure.

1. Acid Precipitation

Cell-free supernatants are subject to acid precipitation. The preferred acid is perchloric acid at 0.6M final concentration. Precipitated protein is removed and the acid soluble fraction which contains the CA 125 activity is neutralized (e.g. with KOH). The acid soluble fraction can then be dialyzed against distilled water and concentrated (e.g. 20X original supernatant volume).

2. Molecular Size Exclusion Chromatography

The acid soluble fraction is submitted to molecular size exclusion chromatography to separate the high molecular weight CA 125 complex from lower molecular weight components. A preferred resin is Sepharose CL-4B resin which retains molecules in the 60 kD to 2,000 kD range. The CA 125 antigen is eluted from this column in the void volume. The antigen can be applied and eluted in phosphate buffered saline (PBS)

3. Treatment with Chaotropic Agent and Molecular Size Exclusion Chromatography

The fraction containing CA125 activity is treated with a chaotropic agent. The chaotropic agent disrupts the high molecular weight CA 125 complex. Urea is preferred, but guanidine-HCl may also be used. Urea treatment is followed by chromatography with a resin that retains molecules in the 200 kD range. A preferred resin is Sepharose CL-6B. The chromatography on Sepharose CL-6B is done with a buffer containing the chaotropic agent (e.g. 6M Urea) and a detergent (e.g. 1% SDS). The eluted fraction can be monitored for CA 125 activity by CA 125 RIA.

4. Affinity Purification

The CA 125 antigen is purified by immunoaffinity chromatography. OC125 is bound to a solid phase (e.g. Protein

A-Sepharose CL-4B resin) and the antigen containing fraction from the prior gel filtration step is passed over the resin under conditions which allow the antigen to bind specifically to the solid phase. The antigen is then eluted with an appropriate eluant such as diethylamine. The preferred immunoaffinity column is prepared essentially by the methods of Schneider *et. al.*, *infra*. OC125 is covalently coupled to Protein A-Sepharose via the coupling agent dimethylpimelidate, a coupling which does not interfere with the activity of the OC125 antibody. Bound antigen is eluted with diethylamine.

In the preferred embodiment, CA 125 antigen is purified from OVCA 433 cell culture supernatants using the procedure described below.

Purification Step 1. Cell culture supernatants from OVCA 433 culture are collected from confluent monolayer cultures. Supernatants are concentrated 10 fold and made 0.6 molar in perchloric acid. Precipitated protein is removed. The acid soluble fraction is neutralized, and then dialyzed against distilled water. The CA 125 reactivity is found in the soluble fraction (95%), while 80% of the protein is removed.

Purification Step 2. The perchloric acid soluble fraction is concentrated and fractionated by gel filtration chromatography using a 3.2 X 85 cm column of Sepharose™ 4B-CL equilibrated in phosphate buffered saline (PBS). CA 125 activity and A₂₈₀ are determined for each fraction. The majority of CA 125 activity is eluted at the void volume (V₀) and a smaller peak which elutes later. This profile indicates the presence of a high molecular weight component (>1000 kDa) and a smaller component (200 to 400kDa). The V₀ fractions contain about 85% of the initial reactivity.

Purification Step 3. The V₀ fraction from the Sepharose 4B-CL column is made 6M in urea and then applied to a 1.2 X 95 cm Sepharose 6B-CL column in 0.1% SDS, 6M urea, 50 mM Tris HCl, pH 8.0. Fractions are collected and assayed for CA 125 reactivity. The CA 125 activity is eluted in two peaks: Peak 1, a minor, high molecular weight component (>1000 kDa) at the void volume and Peak 2, a major, lower molecular weight component (200 to 400 kDa).

Purification Step 4. The material in peak 2 is further purified by immunoaffinity chromatography. An affinity column is prepared by covalently coupling OC125 antibody to a Protein A-Sepharose 4B-CL column according to the method of Schneider *et. al.* The pooled Peak 2, still in 6M urea, 0.1% SDS, is passed over the immunoaffinity column three times. After washing, the antigen is eluted with 50mM diethylamine (DEA) pH 11.5. The eluate is immediately neutralized by collection into a neutralizing buffer, and is then dialyzed against distilled water.

The antigenic activity of CA 125 at each step of this purification has been evaluated using Western blots. Most of the reactivity from Purification Step 1 (PCA extraction) has a very high molecular weight (>1000 kDa), and little or no reactivity is found in the low molecular weight region (<1000 kDa). Analysis of the peak 2 from Purification Step 3 indicates that most of the reactivity is in the 200-400 kDa region. This suggests that the 1000 kDa antigen dissociates into a smaller component. Finally, in Purification Step 4, the antigen eluted from the immunoaffinity column has the 200-400 kDa component with no detectable 1000 kDa reactivity.

To demonstrate that the 200-400 kDa components noted after Steps 3 and 4 were formed by dissociation of the >1000 kDa material, Western blots were performed on a PCA extract, with and without treatment with 6M urea. The untreated PCA extract had a major component of 1000 kDa and a minor 200-400 kDa component. After treatment with 6M urea (45° for 30 minutes), most of the reactivity was found in the 200-400 kDa region.

The isolation procedure of this invention can give a 3900-fold purification relative to starting supernatant material (as determined by activity in units/mg protein in starting material versus final material) The CA 125 antigen species isolated by this procedure is characterized by the following:

It has a molecular weight of about 200 kDa. It is comprised of 24% carbohydrate. The carbohydrate composition is sialic acid, fucose, mannose galactose, N-acetyl-glucosamine, and N-acetyl galactosamine in the ratio 3.6/0.4/3.0/6.6/5.8/2.2.

The region of the OC125 determinant appears to be proteinaceous (See exemplification below).

The isolated immunoreactive 200 KDa species of CA 125 can be used as an immunogen preparation to raise anti-CA 125 antibody. For example, monoclonal anti-CA 125 antibodies can be produced by standard techniques of Kohler and Milstein. A mouse is immunized with the isolated CA 125. Spleen cells are harvested and fused with myeloma cells. Resulting hybridomas can be selected for anti-CA 125 antibody production on the basis of reactivity with isolated CA 125 antigen.

Antibody against CA 125 is useful for diagnosis and therapy of ovarian carcinoma. For example, the antibody can be used in diagnostic tests such as RIAs and ELISAs for the presence of CA 125 in biological fluids. Such antibody can be used in immunohistochemical techniques for identification of tumor techniques for identification of ovarian carcinoma cells. The antibodies may also be used for *in vivo* imaging of ovarian cancer and for immunotherapy of ovarian cancer e.g. passive immuno therapy or immunotoxin therapy.

The isolated CA 125 can also be used to provide an immunoabsorbent for detection of anti CA 125 antibody in the blood. The presence of anti CA 125 antibody may provide an indication of ovarian carcinoma in a patient.

The invention is illustrated further by the following exemplification.

Exemplification

Materials and Methods

5 Materials

The murine monoclonal antibody OC125, produced by hybridomas grown in pristane primed BALB/c mice (Bast, R.C. *et al.* J. Clin. Invest., 68:1331-1337, 1981), was isolated by protein A chromatography (Kabawat, S.E. *et al.* Am. J. Clin. Pathol., 79:98-104, 1983). Serum samples were obtained from women with advanced epithelial ovarian cancer (stage III and IV). Human milk was obtained from a healthy 7-month post partum female. The exoglycosidases and proteases were purchased from Calbiochem, Los Angeles, CA (pronase), and from Sigma, St. Louis, MO (chymotrypsin, trypsin, chondroitinase ABC, α - and β -galactosidase, α -fucosidase, hexaminidase, and neuraminidase). Monoclonal antibody 1116NS 19-9 (Koprowski, H. *et al.* Somat. Cell Genet. 5(6):957-972, 1979; U.S. Patent 4,349,528) was obtained from Dr. Zenon Steplewski, Wistar Institute, Philadelphia, PA. Polyclonal anti-CEA antibody was obtained from Abbott Laboratories, North Chicago, IL. Sepharose CL-4B and CL-6B and Protein A-Sepharose CL-4B were purchased from Pharmacia, Piscataway, NJ. Electrophoresis reagents were purchased from Bio-Rad, Rockville Centre, NY. SeaKem LE agarose was purchased from FMC Corp., Rockland, ME. Fish gelatin was obtained from Norland Products Inc., New Brunswick, NJ. All other reagents were of the highest purity commercially available.

20 Solid-Phase Radioimmunoassays

The simultaneous "sandwich" immunoradiometric assay (IRMA) was used to measure CA 125 activity (Klug, T.L. *et al.* Cancer Res., 44:1048-1053, 1984.) and CA 19-9 activity (Ritts, R.E. *et al.* Int. J. Cancer, 33:339-445, 1984). In the CA 125 IRMA, [125 I]-OC125 (100 μ l, 1×10^5 cpm) was incubated (20 h, 23°C) with polystyrene-immobilized OC125 and sample (100 μ l). The beads were washed (3X) and counted in a gamma counter. Assay kits were manufactured at Centocor, Malvern, PA.

The plate assay was performed using 96 well polyvinyl chloride microtiter plates (Dynatech). The OVCA 433/PCA/4B (see "Isolation of CA 125 from OVCA 433 tissue culture supernatants") fraction was used to coat the wells (100 μ l, 500 units/well). Following the binding of the antigen to the plates (18 h, 4°C), the wells were incubated for 1 h with phosphate buffered saline (PBS) containing 5% (w/v) bovine serum albumin. After the incubation period, the wells were emptied and washed (2X) with PBS. [125 I]-OC125 (20 μ l, 2×10^4 cpm) was then incubated with the immobilized antigen (4 h, 23°C). The wells were subsequently washed (3X) with PBS, cut, and counted in a gamma counter.

As the CA 125 IRMA only detects polyvalent antigens, an inhibition assay was developed to quantitate both mono- and multi-valent antigens. The inhibition assay was performed similarly to the plate assay described above, the only difference being that [125 I]-OC125 (20 μ l, 2×10^4 cpm) was incubated simultaneously (30 μ l, 4 h, 23°C) with various antigen preparations which might inhibit binding of radiolabeled OC125 to the plate. The wells were washed (3X), cut, and counted in a gamma counter. The radioiodinated OC125 used in both the plate and inhibition assays was obtained from Centocor RIA kits.

40 SDS:Polyacrylamide Gel Electrophoresis

Conventional SDS:PAGE was performed essentially according to the method of Laemmli (Laemmli, U.K. Nature 227:680-685, 1970.). The sample buffer did not contain sulfhydryl reducing agents or SDS and was not heated, as the CA 125 antigen was inactivated by these conditions. Some experiments required a polyacrylamide-agarose composite gel for separation of sample components as the CA 125 antigen did not penetrate a conventional 3% (w/v) polyacrylamide gel.

Typically, the composite gels were prepared with 2.5% polyacrylamide and 1.0% agarose. The solutions were heated to 65°C at which time the ammonium persulfate was added. The prewarmed solutions were then immediately poured into the gel apparatus which had been equilibrated at 37°C and the entire apparatus was then cooled at 4°C until the agarose solidified. After overlaying a 2.5% polyacrylamide-1.0% agarose stacking gel at room temperature the samples (300 units/lane) were applied in 10 M urea sample buffer which did not contain sulfhydryl reducing agents or SDS and was not heated. The electrophoresis was performed at 4°C. All buffers used in the preparation and running of the composite gels were also those of Laemmli (See supra.).

55 Immunoblotting

After electrophoresis the proteins were electrophoretically transferred to nitrocellulose (Towbin, *et al.*, Proc. Natl. Acad. Sci. 76:4350-4354, 1979)), immunoblotted with radiolabeled OC125, and autoradiographed. Each immunoblot

contained at least one negative antigen control lane. The electrophoretic transfer was performed at 100 mA overnight. Immunoblotting was accomplished by overlaying the nitrocellulose with radioiodinated OC125 (2 ml, 2×10^6 cpm) in fish gelatin buffer (1% fish gelatin, 50 mM citrate, pH 6.0, 0.05% NP-40) for 6 h. The nitrocellulose sheet was then autoradiographed by exposure to x-ray film with the aid of a Cronex Quanta III fluor screen (Dupont) for 18 h at -80°C .

Fractionation of Human Serum and Human Milk

Whole serum was allowed to clot for 1 h and then centrifuged ($3,000 \times g$, 10 min). A portion (2 ml) of the supernatant was fractionated on a 1.2 x 47 cm Sepharose CL-4B column (human serum/4B) equilibrated in PBS. Fractions (1 ml) containing CA 125 activity, as determined by the CA 125 RIA, were pooled and concentrated. Human milk was defatted by centrifugation ($3,000 \times g$, 1 h) at 10°C . The supernatant was further purified by column chromatography as described above for serum (human milk/4B).

Preparation of CA 125 Antigen Concentrate from OVCA 433 Tissue Culture Supernatant

OVCA 433 human ovarian carcinoma cells were grown in Minimum Essential Medium Eagle supplemented with 2 mM glutamine, 1 mM pyruvate, 1% non-essential amino acids, and 10% heat-inactivated fetal calf serum. T-150 flasks (Costar) were seeded with 1×10^6 cells. Growth was permitted to continue until cells reached confluence at which time the medium was removed. Fresh medium was added and collected at 5-7 day intervals, for a total of 10-12 weeks. OVCA 433 cells appeared to produce the maximum amount of CA 125 antigen in Go growth phase. The concentration of CA 125 antigen produced under these conditions was approximately 1,000 units/ml. Pooled cell supernatants were centrifuged at $10,000 \times g$, filtered through a Sartorius 0.2 micron pore size cascade filter capsule, and concentrated to one-tenth the original volume with an Amicon DC-2 hollow fiber apparatus and filter cartridge (HP 100-200) with a molecular weight cutoff of 100 kDa. The concentrates were stored frozen at -20°C under which conditions the CA 125 activity was stable for at least 12 months.

Isolation of CA 125 from OVCA 433 Tissue Culture Supernatant

The spent tissue culture 10X concentrate of the OVCA 433 cell supernatant was first subjected to perchloric acid (PCA, 0.6 M final concentration) precipitation (Krupey, J. et. al. *J. Exp. Med.* 128:387-398, 1968). The CA 125 activity remained in the PCA soluble fraction and was completely conserved. The acid soluble fraction was neutralized with potassium hydroxide (1.2 M), dialyzed against distilled water (24 h, 4°C), and concentrated to 20X the original supernatant volume. This sample is referred to as OVCA 433/PCA. The OVCA 433/PCA sample (35 ml) was applied to a Sepharose CL-4B column (3.2 x 70 cm) equilibrated in PBS. The fractions (7 ml) which contained CA 125 activity as determined by the CA 125 RIA were pooled and concentrated. This fraction is referred to as OVCA 433/PCA/4B and is used in all experiments except as indicated.

Further fractionation involved treatment of the OVCA 433/PCA/4B fraction with urea (6 M, 30 min, 45°C) and subsequent chromatograph on a Sepharose CL-6B column equilibrated in Tris-urea-SDS (50 mM Tris, 6 M urea, 0.1% SDS, pH 8.0). Final fractionation was accomplished by immunoaffinity chromatography on an OC125-Protein A-Sepharose CL-4B column. The monoclonal antibody OC125 was covalently bound to the Protein A-Sepharose CL-4B column, washed and coupled essentially according to the method of Schneider et al., *J. Biol. Chem.* 257:10766-10169, 1982. Minor modifications included the substitution of citrate buffer (0.05 M, pH 6.0) for Tris-HCl, and taurodeoxycholate (TDC) for deoxycholate (DDC). Repeated passes (3X) over the affinity column of the CA 125 reactive lower molecular weight fraction from the Sepharose CL-4B column in 0.1% SDS and 6 M urea gave greater than 80% binding of the CA 125 activity. Elution of the CA 125 antigen from the column was accomplished with the use of diethylamine (DEA) (50 mM, pH 11.3). This affinity purified antigen is referred to as OVCA 433/4B/DEA.

Density Gradient Ultracentrifugation

Ultracentrifugation of the CA 125 antigen isolated either from human serum, human milk, or from the OVCA 433 tissue culture supernatant after chromatography on Sepharose CL-4B was performed in a cesium chloride isopycnic density gradient in PBS (2.276 g of CsCl dissolved in 3.414 ml of PBS). The buoyant density of B-galactosidase was determined as a standard. Fractions (0.2 ml) were assayed for B-galactosidase activity following equilibrium by the method of Miller (*Experiments in Molecular Genetics*, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1972). Gradients were formed by ultracentrifugation in a Beckman SW50.1 rotor (33,000 rpm, 68 h, 10°C) under conditions which have been described (See, Magnani, J.L. et. al., *Cancer Res.* 43:4589-5492, 1983). Fractions (0.2 ml) were collected and assayed for activity using the CA 125 RIA described above. The density of each fraction was determined by weighing a known volume.

Chemical Treatments

Periodate oxidation of the CA 125 antigen was accomplished with 0, 0.1, 1.0, 10.0 and 100 mM periodate in acetate buffer (pH 4.5, 50 mM, 4°C) in the dark according to Stahl *et al.*, *Proc. Natl. Acad. Sci.* **73**:4045-4049, 1976. Reduction and alkylation were performed according to methods described elsewhere. See, Glazer, A.N. *et al.*, *Chemical Modifications of Proteins*. In: T.S. Work and E. Wbrk/Eds) *Laboratory Techniques in Biochemistry and Molecular Biology*, p. 104, New York:Elsevier Publishing Co., 1975. Reduction was accomplished with DTT (10 mM, 50 mM Tris, pH 8.1, 4 h, 45°C) either in the presence or absence of guanidine-HCl (6 M). Alkylation was performed with iodoacetic acid (20 mM, 30 min) after the samples had been cooled to room temperature. The samples were immediately dialyzed (4°C, 18 h) against distilled water.

Exoglycosidase Treatments

Exoglycosidase digestions were performed in acetate buffer (.01 M, pH 4.5, 48 h, 37°C). Unit values of the exoglycosidases were chosen in order to ensure complete digestion of the oligosaccharide residues within an appropriate time frame. All exoglycosidase digestions were performed under conditions whereby the appropriate substrates were shown to be completely hydrolyzed as detected by thin layer chromatography. CA 125 activity following treatment was measured both by the CA 125 RIA and by the plate assay as previously described.

Exhaustive Protease Digestion

The various protease digestions were performed in Tris-HCl buffer (0.2 M, pH 8.0, 10 mM calcium chloride). The proteases trypsin, chymotrypsin, and pronase (2% w/v, 50 ul) were added to wells containing antigen and allowed to incubate (48 h, 37°C). Protease digestions were performed under conditions which caused hydrolysis of albumin as detected by thin layer chromatography. Samples were assayed for CA 125 activity by both the CA 125 RIA and the plate assay as described above.

Amino Acid Analysis

Samples of OVCA 433/4B/DEA were dissolved in 6N HCl containing 0.1% phenol, sealed under vacuum, and hydrolyzed for 24 h at 110°C. Amino acids were derivatized with phenylisothiocyanate (PITC) and derivatized PTC-amino acids separated and quantitated by HPLC using the Waters PICO-TAG column and elution conditions See Bidlingmeyer, B.A. *et al.* *J. Chromatogr.*, **336**:93-104 (1984).

Carbohydrate Composition

Samples of the same lot of OVCA 433/4B/DEA that had been subjected to amino acid quantitation were subjected to carbohydrate compositional analysis as described by Yang and Hakomori, *J. Biol. Chem.* **246**:1192-1200, 1971. The samples were subjected to acetolysis, followed by hydrolysis and reduction. The resultant alditols were per-O-acetylated with acetic anhydride. Quantitation of sialic acid was accomplished by trimethylsilyl derivatization (TMS). Laine, R. A. *et al.*, *Meth. Enzymol.* **28**:159-167, 1972. Both the alditol acetate and the TMS-methyl glycosides were separated by a Hewlett Packard 5790 gas chromatograph and identified by a Hewlett Packard 5790 mass selective detector (MSD).

Results

Physical and Immunological Characteristics of the CA 125 Antigen

The antigen isolated from OVCA 433 and from human ovarian cancer patient serum by perchloric acid precipitation elutes primarily in the void volume of a Sepharose CL-4B column (Figure 1). In addition, a smaller peak of CA 125 activity elutes from the column later indicating a much lower molecular weight. These peaks of CA 125 activity correspond to molecular masses of greater than 1,000 and about 200-400 kDa. The CA 125 antigen elution pattern of human milk is similar to that shown for OVCA 433 and human cancer patient serum.

In an effort to compare the physical characteristics of the antigen isolated from OVCA 433 cell supernatants, ovarian cancer patient serum, and human milk, a buoyant density was determined for each (Figure 2). The average buoyant density of the antigen isolated from OVCA 433 after passage over a Sepharose CL-4B column (OVCA 433/4B) is approximately 1.42 g/ml whereas the buoyant densities of the patient serum/4B and the milk/4B are 1.46 and 1.39 g/ml, respectively. As a standard, the buoyant density of (β)-galactosidase was determined and found to be 1.32 g/ml.

This agrees well with the published value of 1.316 g/ml Costini, N.V. et. al., *J. Biol. Chem.* **254**:11242-11246, 1979. Figure 3 compares the electrophoretic mobility of immunoreactive species from OVCA 433/4B, human milk/4B, and ovarian cancer serum/4B on a composite 2.5% polyacrylamide/1.0% agarose gel. The samples were applied in 10 M urea sample buffer which did not contain DTT or SDS and was not heated. The immunoblotted OC125 reactive antigen from each of the sources is present as high molecular mass complexes of between 200 and 1,000 kDa with similar electrophoretic profiles. This data, which suggests multiple aggregated states of the CA 125 antigenic complex, correlates well with the Sepharose CL-4B elution profile shown in Figure 1. Both experiments indicate that antigen exists as a high molecular mass species of greater than 1,000 kDa and lower molecular mass species of approximately 200-600 kDa.

When the OVCA 433/PCA/4B fraction is subjected to SDS:PAGE electrophoresis using a 3-12% polyacrylamide gradient gel followed by immunoblotting (Figure 4), the lane which is reactive with radioiodinated monoclonal antibody OC125 give rise to a band of greater than 1,000 kDa molecular mass and a lower molecular mass band of approximately 400 kDa. The sample buffer used contained only 10% glycerol, 0.08 M Tris, pH 6.8, and bromophenol blue. After overlaying the adjacent lane with radioiodinated monoclonal antibody 19-9, which recognizes the sialylated lacto-N-fucopentaose II carbohydrate determinant, only the higher molecular mass band is observed. The lane which is overlaid with radioiodinated anti-CEA does not shown any immunoreactivity. Furthermore, Western blots using monoclonal antibody 19-9 as an overlay with the OC125 affinity purified CA 125 antigen (OVCA 433/4B/DEA) fraction does not give rise to any bands. Also, there is no CA 19-9 activity present when measured by the CA 19-9 RIA (data not shown). This result clearly demonstrates that the antigenic determinants CA 125 and CA 19-9 are located on the same high molecular mass glycoprotein complex, but the CA 125 and CA 19-9 determinants are not present on the same glycoprotein molecule.

The results of Sepharose CL-4B column chromatography and of SDS:PAGE analysis suggests that the lower molecular weight material was probably derived from the higher molecular weight species. Attempts to disaggregate the high molecular weight material with both ionic (SDS) and non-ionic (NP-40) detergents proved futile. However, treatment of the pooled and concentrated void volume fraction of the Sepharose CL-4B column of OVCA 433/PCA with 6 M urea for 30 min at 45°C, followed by column chromatography on Sepharose CL-6B in 0.1% SDS and 6 M urea yields two peaks, as shown in Figure 5. Following this step, the majority (80%) of the CA 125 activity is found associated with a much lower molecular mass peak of approximately 200 kDa. This is verified by electrophoresis and immunoblotting of fractions from the Sepharose CL-6B column chromatography (Fig. 5). Some of the antigen still remains in the high molecular mass aggregated form.

Immunoaffinity Purification of the CA 125 Antigen from OVCA 433 Calls. Sepharose CL-4B column chromatography followed by treatment with 6 M urea and heat with subsequent column chromatography on Sepharose CL-6B in the presence of 6 M urea and 0.1% SDS (Fig. 5) results in a 1,400-fold purification of CA 125 antigen from OVCA 433 supernatants (data not shown). This preparation has a specific activity of 117 units CA 125/ug of protein. The specific activity is determined by measuring the CA 125 activity using the Centocor CA 125 RIA kit and determining the amount of protein by amino acid analysis on this same lot of purified CA 125 antigen. Final fractionation of the antigen is accomplished by immunoaffinity on an immobilized OC125-Protein A-Sepharose CL-4B column. The antigen which eluted from the column with diethylamine (DEA) has a specific activity of 317 units CA 125/ug of protein.

Samples of antigen eluting from a Sepharose CL-4B column and from an OC125 immunoaffinity column were subjected to density gradient ultracentrifugation. This procedure reveals different average buoyant densities for the two antigen preparations (Fig. 6). The more highly purified DEA eluate has a buoyant density of approximately 1.36 g/ml whereas the buoyant density of the OVCA 433/4B is approximately 1.42 g/ml. This suggests that the less pure antigen is associated with more highly glycosylated proteins which would result in the polydisperse nature of the buoyant density profile as well as the higher average buoyant density observed.

Carbohydrate Composition of the CA 125 Antigen Isolated by Affinity Chromatography. Preliminary carbohydrate composition of OVCA 433/4B/DEA reveals that sialic acid, fucose, mannose, galactose, N-acetyl glucosamine, and N-acetyl galactosamine are present in the ratio 3.6:0.40:3.0:6.6:5.8:2.2, respectively (data not shown). This data suggests that there are both N- and O-linked oligosaccharides present. In addition, this immunopurified CA 125 antigen is found to contain 24% carbohydrate, by mass, in close agreement with that calculated from its buoyant density of 1.36 g/ml. Therefore, the CA 125 antigen is not a typical mucin and does not have a significant amount, if any, of lipid associated with it.

Nature of the CA 125 Determinant. The nature of the CA 125 determinant was investigated using a number of chemical and physical treatments, as well as exhaustive exoglycosidase and protease digestions of the antigen. Periodate oxidation (Table I) of the CA 125 immunoreactive antigen isolated from OVCA 433/4B and from human milk/4B has no effect on activity at periodate concentrations and reaction times that totally destroyed activity of the CA 19-9 carbohydrate determinant, the sialylated lacto-N-fucopentaose II. In fact, at the lowest periodate concentrations which destroyed CA 19-9 activity (0.1 mM) there actually appears to be an increase in CA 125 activity. Only at very high concentrations of periodate (100 mM) or at very long reaction times (24 h) is there a significant decrease in CA 125

activity, which is likely due to non-specific oxidation of the antigen protein backbone.

TABLE I

EFFECT OF PERIODATE OXIDATION ON CA 125 ACTIVITY AT VARIOUS CONCENTRATIONS AND REACTION TIMES.

SAMPLE	PERIODATE CONCENTRATION (mM)	CA 125 Activity (% Remaining) TIME (h)			
		0	3	6	24
OVCA 433/PCA/4B	0	100	99	102	102
	0.1	100	110	115	92
	1.0	100	134	144	121
	10.0	100	151	135	119
	100.0	100	72	48	40
Human milk/4B	0	100	101	103	98
	0.1	100	103	93	97
	1.0	100	107	110	84
	10.0	100	111	69	50
	100.0	100	7	5	5
Positive Control 19-9 cGP	0	100	100	97	99
	0.1	100	16	11	5
	1.0	100	10	5	5

Chemical and physical treatments (Table II) which denature most proteins, that is, reduction and alkylation in 6 M guanidine-HCl, 8 M urea, and boiling all reduce the CA 125 immunoreactivity substantially. Reduction alone, however, does not seem to affect CA 125 immunoreactivity. Thus, the decrease in activity observed with either reduction of alkylation in the presence of guanidine-HCl is mainly the result of guanidine-HCl acting on the antigen. There is almost complete loss of activity with reduction and alkylation in the presence of guanidine-HCl. In addition, neither the anionic detergent SDS nor the non-ionic detergent NP-40 affects the CA 125 immunoreactivity.

TABLE II

THE EFFECT OF CHEMICAL TREATMENTS ON CA 125 ACTIVITY

Treatment	CA 125 Activity (% Remaining)		
	Plate Assay	Inhibition Assay	IRMA (Sandwich)
Control	100	100	100
Guanidine-HCl(6M, 45° 4h)	ND	20	49
Reduction (10 mM DTT, 4h, 45°C)	ND	73	98
Reduction in guanidine-HCl	ND	40	20
Alkylation (20mM iodoacetic acid, 30 min, 23°C)	ND	31	82
Alkylation in guanidine-HCl	ND	40	29
Reduction and alkylation	ND	53	51
Reduction and alkylation in guanidine-HCl	5	12	5-7
Urea (8M, 24h, 4°C)	100	100	100
Urea (8M, 24h, 45°C)	15	10	0
Heat (100°C, 20 min.)	0	0	0
SDS (2%)	100	ND	100
NP-40 (10%)	100	ND	100
ND designates assay was <i>not</i> done.			

Various combinations of exoglycosidase treatments were performed on the CA 125 antigen (Table III). The solid-phase IRMA indicates only slight losses of CA 125 immunoreactivity either with α -galactosidase and/or β -galactosidase treatments. On the other hand, no loss of immunoreactivity is demonstrated using the plate assay. In fact, there is an

increase in the ability of the immobilized antigen to bind radiolabeled OC125 antibody following most of the exoglycosidase treatments. This result corroborates that obtained with periodate oxidation, that is, removal of terminal carbohydrate moieties may actually increase access of OC125 to the CA 125 determinant.

Finally, exhaustive protease digestion with pronase, trypsin, or chymotrypsin causes complete loss of antigenic activity as measured with either the IRMA or the plate assays (Table III).

TABLE III

THE EFFECT OF ENZYMATIC DIGESTION ON CA 125 ANTIGEN ACTIVITY ISOLATED FROM OVCA 433		
Exoglycosidase Treatment	CA 125 Activity (% Remaining)	
	IRMA	Plate Assay
Control	100	100
Neuraminidase (N)	96	126
N + α -Fucosidase (F)	106	128
N + F + β -Galactosidase (β G)	96	129
N + F + β G + Hexosaminidase	109	123
α -galactosidase	94	117
α + β -galactosidase	88	116
Chondroitinase ABC	93	94
Exhaustive Protease Treatment	IRMA	Plate Assay
Pronase	0	0
Trypsin	0	0
Chymotrypsin	0	0

Discussion

The murine monoclonal antibody OC125 recognizes a human ovarian carcinoma-associated antigenic determinant (CA 125). We have isolated glycoprotein complexes from the ovarian cancer cell line OVCA 433, human serum, and human milk all of which express CA 125 determinant activity. In addition, we have evidence of CA 125 activity in seminal plasma which is in contrast to the observations of de Bruijn *et al. supra*. Chemical treatment and chromatography of the high molecular weight complex isolated from OVCA 433 cell supernatants gave rise to a 200 kDa immunoreactive species. It is possible, however, that the actual protein which expressed the antigenic determinant may be of still lower molecular weight. Further attempts to isolate a lower molecular weight immunoreactive species have thus far proven ineffective. Moreover, the isolation scheme described here does not give rise to a completely homogenous and pure species.

The antigen expressing the CA 125 determinant isolated from several sources exists as a high molecular weight glycoprotein complex with an average buoyant density of between 1.36 and 1.46 g/ml. Moreover, these average densities indicated that each of the antigens isolated from three sources may have had a slightly different protein and carbohydrate composition. If a mucin is defined as a high molecular weight glycoprotein composed of 50% or more carbohydrate with a majority of O-linked oligosaccharides containing little or no N-linked chains, then the CA 125 antigen is not a typical mucin. This conclusion is based on the CA 125 carbohydrate composition of 24%, the high amount of mannose present, the majority of N-linked oligosaccharides, and the CA 125 antigen buoyant density. The average buoyant density of unglycosylated protein is between 1.25 and 1.35 g/ml, while the average buoyant density of mucins is approximately 1.50 g/ml. This finding is in contrast to that reported for other epithelial tumor-associated antigens recognized by monoclonal antibodies such as 19-9 (Magnani *et al. supra*, *J. Biol. Chem.* **257**:14365-14369, 1982), B72.3 (Johnson, V.G. *et al.*, *Cancer Res.* **45**:850-857, 1986) DU-PAN-2 (Lan, M.S. *et al.*, *Cancer Res.* **45**:305-310, 1985), and F36/22 (Croghan, G.A. *et al.*, *Cancer Res.* **43**:4980-5988, 1983), all of which have been classified as high molecular weight mucin-like glycoproteins based on their higher buoyant densities.

The higher molecular weight antigen complex isolated from the supernatant of OVCA 433 was reactive with the monoclonal antibody 19-9 (Magnani *et al. supra*), suggesting that the CA 19-9 determinant is present on this complex. However, we have clearly shown by electrophoresis and immunoblotting that the CA 19-9 and the CA 125 determinants were not located on the same glycoprotein since the OC125 immunoaffinity purified CA 125 antigen showed no reactivity with the monoclonal antibody 19-9. This observation is contrary to that suggested by Hanisch *et al. supra* who had isolated both CA 19-9 and CA 125 activity from human milk.

Chemical and physical treatments of CA 125 antigen were undertaken to better understand the nature of the

antigenic determinant recognized by the monoclonal antibody OC125. Periodate oxidation of CA 125 reduced the immunoreactivity only at high concentrations of periodate or with prolonged reaction times. In fact, the activity of the antigen actually increased at concentrations and reaction times which caused total loss of immunoreactivity of the CA 19-9 determinant. Non-specific oxidation of the protein backbone probably caused the loss of CA 125 activity at higher concentrations of periodate. During CA 125 antigen purification, there was a loss of 82% of the original activity following urea and heat treatment. This apparent loss in activity was most likely due to breakdown of antigen complex to a less aggregated form or to partial denaturation of the antigen. A lower aggregated state may lead to a lower unit value as the CA 125 RIA is sensitive to CA 125 antigen valency, i.e. the number of OC125 binding sites per antigen molecule.

The observations of Hanisch *et al. supra* which suggested that the CA 125 determinant is carbohydrate in nature were based on two criteria; its sensitivity to periodate oxidation (at a concentration of 100 mM and a reaction time of (18 h), and its loss of activity under conditions which would selectively cleave N-acetylneuraminic acid (pH 3.3, 100°C). Their results also indicated that neuraminidase treatment alone caused only slight reduction of immunoreactivity even though approximately 97% of the mucin-linked sialic acid was cleaved. Our results clearly show that concentrations of periodate sufficient to oxidize carbohydrates do not affect CA 125 activity. It is not surprising, therefore, that pH 3.3 at 100°C destroyed CA 125 antigenic activity. In addition, greater than 95% of the activity was lost upon reduction and alkylation treatment in the presence of guanidine-HCl. Lastly, exoglycosidase treatments actually caused an increase in CA 125 activity while antigen activity was completely eradicated with exhaustive protease digestion. These data strongly suggest that the CA 125 determinant is proteinaceous in nature, or at the very least, is protein associated with carbohydrate in a conformationally dependent epitope. This may explain the similarity of the antigen isolated from the various sources such as human serum, OVCA 433, and human milk. A peptide determinant would be expected to be more highly conserved than a carbohydrate determinant, that is, a protein sequence is more likely to be associated with a single unique protein, whereas a carbohydrate structure may exist on several different proteins. These results may not be completely unique as the nature of the tumor associated glycoprotein epitope (TAG-72) recognized by the monoclonal antibody B72.3 seemed to indicate protein, in addition to carbohydrate, as forming part of the conformationally dependent TAG-72 determinant.

Claims

1. An isolated immunoreactive species of the antigen CA 125 which is a glycoprotein having a molecular weight of about 200 kDa as determined by SDS:PAGE chromatography, having a carbohydrate composition of about 24%, by mass, and which is reactive with monoclonal antibody OC-125 but is not reactive with anti-CA-19-9 antibodies.
2. A method of isolating the antigen CA 125 as defined in Claim 1 from ovarian carcinoma cells, comprising the steps of:
 - a) obtaining a cell culture medium from a culture of ovarian carcinoma cells which shed CA 125 into the medium;
 - b) subjecting the medium to acid precipitation (e.g. with perchloric acid) to provide an acid soluble and an acid insoluble fraction;
 - c) recovering and neutralizing the acid soluble fraction;
 - d) separating the CA 125 species in the acid soluble fraction from lower molecular weight components of the fraction by molecular exclusion chromatography (performed e.g. on Sepharose 4B-CL resin) and recovering the CA 125 species;
 - e) treating the recovered CA 125 species with a chaotropic agent to disrupt high molecular weight CA 125 species;
 - f) separating the lower molecular weight CA 125 species by molecular exclusion chromatography (performed e.g. on Sepharose 6B resin) in the presence of the chaotropic agent;
 - g) recovering the eluted fraction containing CA 125 species;
 - h) contacting the CA 125 species with an immunoabsorbent comprising an antibody which binds CA 125 coupled to a resin under conditions which permit selective adsorption of CA 125 by the immunoabsorbent; and
 - i) recovering the 200 kDa CA 125 from the immunoabsorbent.
3. An immunoreactive species of the antigen CA 125 isolated from ovarian carcinoma cells by the method of Claim 2, having the following characteristics:
 - a) a molecular weight of about 200 kDa;
 - b) a buoyant density of about 1.36 g/ml;
 - c) a carbohydrate composition of about 24% by mass;

- d) a carbohydrate composition of sialic acid, fucose, mannose, galactose, N-acetyl glucosamine, and n-acetyl galactosamine in a ratio of about 3.6/0.4/3.0/6.5/5.8/2.2; and
- e) reactivity with the antibody OC-125 but not with anti-CA-19-9 antibody.

- 5 4. A method of Claim 2, wherein the chaotropic agent is urea or guanidine-HCl.
5. A method of Claim 4, wherein the chaotropic agent is separated from the CA 125 species by dialysis after the molecular size exclusion chromatography of step f.
- 10 6. A method of Claim 5, wherein the antibody which binds to CA 125 is the OC 125 antibody.
7. A method of isolating the antigen CA 125 as defined in Claim 1 from ovarian carcinoma cells, comprising the steps of:
 - 15 a) obtaining a cell-free supernatant from a culture of ovarian carcinoma cells;
 - b) acidifying the supernatant (e.g. with perchloric acid) to precipitate protein;
 - c) separating the precipitated protein from the acid soluble fraction of the supernatant;
 - d) neutralizing the soluble fraction;
 - e) separating the high molecular weight (1000kD) CA 125 from the smaller molecular weight CA 125 species and from other components in the soluble fraction by molecular size exclusion chromatography (performed e.
 - 20 g. on Sepharose 4B-CL resin);
 - f) treating the high molecular weight CA 125 species with urea (e.g. about 6 molar urea) to disrupt the high molecular weight species;
 - g) separating the CA 125 species by molecular exclusion chromatography on a resin (e.g. Sepharose B resin) which retains molecules in the 200kD range in the presence of urea; and
 - 25 h) immunopurifying the CA 125 species.
8. A method of Claim 7, wherein the ovarian carcinoma cells are selected from the group consisting of OVCA 433, NIH; OVCAR-3, SK-OV-3, CAOV-3 and CAOV-4.
- 30 9. A method of isolating the antigen CA 125 as defined in Claim 1 from ovarian carcinoma cells, comprising the steps of:
 - a) obtaining a cell free supernatant from a culture of ovarian carcinoma cells which shed CA 125 into the culture medium;
 - 35 b) acidifying the supernatant with perchloric acid to precipitate protein;
 - c) removing precipitated protein and neutralizing the acid soluble fraction;
 - d) submitting the neutralized acid soluble fraction to molecular size exclusion chromatography on Sepharose CL-4B resin and recovering from the column the fraction (void volume) containing CA 125 activity;
 - 40 e) treating the fraction containing CA 125 activity with urea at about 6M;
 - f) submitting the urea treated fraction to molecular size exclusion chromatography on Sepharose CL-6B in a buffer controlled Urea 6M and about 1% SDS and recovering the eluted fraction containing CA 125 activity;
 - g) removing the urea from the recovered fraction;
 - h) applying the fraction to an immunoaffinity column comprising OC 125 antibody coupled to protein A Sepharose via dimethylpimelidate;
 - 45 i) eluting the CA 125 from the immunoaffinity column with diethylamine.
10. An immunoabsorbent for specific adsorption of antibody reactive with CA 125, comprising an isolated immunoreactive species of CA 125 antigen of Claim 1.
- 50 11. An immunogen composition for immunizing an animal against CA 125 antigen comprising isolated CA 125 antigen of Claim 1 in a physiologically acceptable vehicle.
12. An immunogen composition of Claim 11, wherein the isolated CA 125 antigen is an immunoreactive species of CA 125 having a molecular weight of about 200 kDa.
- 55 13. The use of the immunogen of Claim 11 for production of antibody against CA 125.

Patentansprüche

1. Isolierte immunoreaktive Spezies des Antigens CA 125, welche ein Glykoprotein ist mit einem Molekulargewicht von ca. 200 kDa, bestimmt durch SDS:PAGE Chromatographie, eine Carbohydrazusammensetzung von ca. 24 Masse-% hat und mit dem monoklonalen Antikörper OC-125, aber nicht mit Anti-CA-19-9 Antikörpern reagiert.

2. Verfahren zur Isolierung des Antigens CA 125 nach Anspruch 1 aus Eierstockkrebszellen in folgenden Schritten:

- a) Verschaffen eines Zellkulturmediums aus einer Kultur von Eierstockkrebszellen, die CA 125 in das Medium absondern;
- b) Säurefällung des Mediums (z.B. mit Perchlorsäure) um eine säurelösliche und eine säureunlösliche Fraktion zu erhalten;
- c) Rückgewinnung und Neutralisation der säurelöslichen Fraktion;
- d) Abtrennung der CA 125 Spezies in der säurelöslichen Fraktion von niedermolekularen Bestandteilen der Fraktion durch Molekülgrößenausschluß-Chromatographie (z. B. an Sepharose 4B-CL Harz) und Rückgewinnung der CA 125 Spezies;
- e) Behandlung der zurückgewonnenen CA 125 Spezies mit einem chaotropen Mittel, um CA 125 Spezies mit hohem Molekulargewicht zu spalten;
- f) Abtrennung der CA 125 Spezies mit niedrigem Molekulargewicht durch Molekülgrößenausschluß-Chromatographie (z. B. an Sepharose 6B Harz) in Gegenwart des chaotropen Mittels;
- g) Gewinnung der eluierten, CA 125-haltigen Fraktion;
- h) Kontaktierung der CA 125 Spezies mit einem Immunoabsorbens, das einen Antikörper enthält, der an ein Harz gekoppeltes CA 125 unter Bedingungen bindet, die eine selektive Adsorption des CA 125 durch das Immunoabsorbens erlauben; und
- i) Gewinnung des 200 kDa CA 125 aus dem Immunoabsorbens.

3. Immunoreaktive Spezies des Antigens CA 125, das nach der in Anspruch 2 beschriebenen Methode aus Eierstockkrebszellen isoliert wurde, mit folgenden Merkmalen:

- a) Molekulargewicht von ca. 200 kDa;
- b) Dichte des Überstands von ca. 1,36 g/ml;
- c) Carbohydrazusammensetzung von ca. 24 Masse-%;
- d) Carbohydrazusammensetzung aus Speichelsäure, Fucose, Mannose, Galactose, N-Acetylglukosamin und n-Acetylgalactosamin in einem Verhältnis von ca. 3,6 : 0,4 : 3,0 : 6,5 : 5,8 : 2,2; und
- e) Reaktivität gegenüber dem Antikörper OC-125, nicht jedoch gegenüber dem CA-19-9 Antikörper.

4. Verfahren gemäß Anspruch 2, wobei das chaotrope Mittel Harnstoff oder Guanidin-HCl ist.

5. Verfahren gemäß Anspruch 4, wobei das chaotrope Mittel von dem CA 125 Antigen im Anschluß an die Molekülgrößenausschluß-Chromatographie gemäß Schritt (f) durch Dialyse abgetrennt wird.

6. Methode gemäß Anspruch 5, wobei der sich an das CA125 bindende Antikörper OC 125 ist.

7. Verfahren zur Isolierung des Antigens CA 125 aus Eierstockkrebszellen gemäß Anspruch 1 in folgenden Schritten:

- a) Gewinnung eines zellfreien Überstands aus einer Eierstockkrebszellenkultur;
- b) Ansäuern des Überstands (z.B. mit Perchlorsäure) zur Fällung des Proteins;
- c) Abtrennung des gefällten Proteins aus der säurelöslichen Fraktion des Überstands;
- d) Neutralisation der löslichen Fraktion;
- e) Abtrennung des CA 125 von hohem Molekulargewicht (1000kD) von der CA 125 Spezies mit niedrigerem Molekulargewicht und anderen Komponenten in der löslichen Fraktion durch Molekülgrößenausschluß-Chromatographie (z.B. an Sepharose 4B-CL Harz);
- f) Behandlung der CA 125 Spezies mit höherem Molekulargewicht mit Harnstoff (z.B. ca. sechsmolarem Harnstoff) zur Spaltung der hochmolekularen Spezies;
- g) Abtrennung der CA 125 Spezies durch Molekülgrößenausschluß-Chromatographie an einem Harz (z.B. Sepharose B), das in Gegenwart von Harnstoff Moleküle im Bereich von 200 kD zurückhält; und
- h) Immunoreinigung der CA 125 Spezies.

8. Verfahren gemäß Anspruch 7, wobei die Eierstockkrebszellen aus einer Gruppe bestehend aus OVCA 433, NIH, OVCAR-3, SK-OV-3, CAOV-3 und CAOV-4 ausgewählt werden.

9. Verfahren zur Isolierung des Antigens CA 125 nach Anspruch 1 aus Eierstockkrebszellen in folgenden Schritten:

- a) Gewinnen eines zellfreien Überstands aus einer Kultur von Eierstockkrebszellen, die CA 125 in das KulturmEDIUM absondern;
- b) Ansäuern des Überstands mit Perchlorsäure zur Fällung des Proteins;
- c) Entfernen gefällten Proteins und Neutralisation der säurelöslichen Fraktion;
- d) MolekülgröÙenausschluß-Chromatographie der neutralisierten säurelöslichen Fraktion an Sepharose CL-4B Harz und Gewinnung der Fraktion (Leervolumen) mit CA-125 Aktivität von der Säule;
- e) Behandeln der CA 125-aktiven Fraktion mit Harnstoff von ca. 6 M;
- f) MolekülgröÙenausschluß-Chromatographie der mit Harnstoff behandelten Fraktion an Sepharose CL-6B in gepuffertem Harnstoff 6 M und ca. 1 % SDS sowie Gewinnung der eluierten Fraktion mit CA 125 Aktivität;
- g) Entfernen des Harnstoffs aus der zurückgewonnenen Fraktion;
- h) Aufgeben der Fraktion auf eine immunoaffine Säule mit dem an Protein A Sepharose gebundenen Antikörper OC 125 via Dimethylpimelimidat;
- i) Eluieren der CA 125 Spezies von der immunoaffinen Säule mit Diethylamin.

10. Immunoadsorbens zur spezifischen Adsorption eines mit CA 125 reaktiven Antikörpers, das eine isolierte immunoreaktive Spezies des Antigens CA 125 gemäß Anspruch 1 aufweist.

11. Immunogenzusammensetzung zur Immunisierung eines Tieres gegen das Antigen CA 125, die das isolierte CA 125 Antigen gemäß Anspruch 1 in einem physiologisch akzeptablem Trägermittel enthält.

12. Immunogenzusammensetzung gemäß Anspruch 11, worin das isolierte CA 125 Antigen eine immunoreaktive Spezies des CA 125 mit einem Molekulargewicht von ca. 200 kDa ist.

13. Verwendung des Immunogens gemäß Anspruch 11 zur Herstellung eines Antikörpers gegen CA 125.

Revendications

1. Espèce immunoréactive isolée de l'antigène CA 125, qui est une glycoprotéine ayant un poids moléculaire d'environ 200 kd (déterminé par chromatographie SDS-PAGE), ayant une teneur en glucides d'environ 24% en masse et qui réagit avec l'anticorps monoclonal OC-125, mais ne réagit pas avec l'anticorps anti-CA-19-9.

2. Procédé d'isolement de l'antigène CA 125 de 200 kd à partir de cellules de carcinome ovarien, comprenant les étapes consistant :

- a) à recueillir le milieu de culture cellulaire d'une culture de cellules de carcinome ovarien qui déversent le CA 125 dans le milieu;
- b) à soumettre le milieu à une précipitation par acide (par exemple par de l'acide perchlorique) pour former une fraction soluble dans l'acide et une fraction insoluble dans l'acide;
- c) à récupérer et à neutraliser la fraction soluble dans l'acide;
- d) à séparer l'espèce de CA 125 contenue dans la fraction soluble dans l'acide d'avec des composants de plus bas poids moléculaire de cette fraction, par chromatographie d'exclusion moléculaire (effectuée par exemple sur résine Sepharose 4B-CL) et à récupérer l'espèce de CA 125;
- e) à traiter l'espèce de CA 125 récupérée par un agent chaotrope pour dissocier l'espèce de CA 125 de poids moléculaire élevé;
- f) à séparer l'espèce de CA 125 de plus bas poids moléculaire par chromatographie d'exclusion moléculaire (effectuée par exemple sur résine de Sepharose 6B) en présence de l'agent chaotrope;
- g) à récupérer la fraction éluée contenant l'espèce de CA 125;
- h) à mettre l'espèce de CA 125 en contact avec un immunoadsorbant comprenant un anticorps qui se lie à CA 125, associé à une résine, dans des conditions qui permettent une adsorption sélective de CA 125 par l'immunoadsorbant; et
- i) à récupérer le CA 125 de 200 kd à partir de l'immunoadsorbant.

3. Espèce immunoréactive de l'antigène CA 125, isolée de cellules de carcinome ovarien par le procédé selon la revendication 2, présentant les caractéristiques suivantes :

- a) un poids moléculaire d'environ 200 kd;
- b) une masse volumique flottante d'environ 1,36 g/ml;
- c) une teneur en glucides d'environ 24% en masse;
- d) une composition de glucides comprenant de l'acide sialique, du fucose, du mannose, du galactose, de la N-acétylglucosamine et de la N-acétylgalactosamine dans un rapport d'environ 3,6/0,4/3,0/6,5/5,8/2,2; et
- e) une réactivité avec l'anticorps OC-125, mais non avec l'anticorps anti-CA-19-9.

4. Procédé selon la revendication 2, dans lequel l'agent chaotrope est l'urée ou la guanidine-HCl.

5. Procédé selon la revendication 4, dans lequel l'agent chaotrope est séparé de l'espèce de CA 125 par dialyse après la chromatographie d'exclusion de taille moléculaire de l'étape f).

6. Procédé selon la revendication 5, dans lequel l'anticorps qui se lie à CA 125 est l'anticorps OC-125.

7. Procédé d'isolement de l'antigène CA 125, tel que défini dans la revendication 1, à partir de cellules de carcinome ovarien, comprenant les étapes consistant :

- a) à recueillir un liquide surnageant dépourvu de cellules à partir d'une culture de cellules de carcinome ovarien;
- b) à acidifier le liquide surnageant (par exemple avec de l'acide perchlorique) pour précipiter la protéine;
- c) à séparer la protéine précipitée d'avec la fraction soluble dans l'acide du liquide surnageant;
- d) à neutraliser la fraction soluble;
- e) à séparer le CA 125 de poids moléculaire élevé (1000 kd) de l'espèce de CA 125 de plus bas poids moléculaire et d'autres composants contenus dans la fraction soluble, par chromatographie d'exclusion de taille moléculaire (effectuée par exemple sur résine Sepharose 4B-CL);
- f) à traiter l'espèce de CA 125 de poids moléculaire 0 élevé par de l'urée (par exemple de l'urée 6 molaire environ) pour dissocier l'espèce de poids moléculaire élevé;
- g) à séparer l'espèce de CA 125 par chromatographie d'exclusion moléculaire sur une résine (par exemple la résine Sepharose B) qui retient les molécules dans la gamme de 200 kd, en présence d'urée;
- h) à immunopurifier l'espèce de CA 125.

8. Procédé selon la revendication 7, dans lequel les cellules de carcinome ovarien sont choisies dans le groupe constitué par OVCA 433, NIH: OVCAR-3, SK-OV-3, CAO-V-3 et CAO-V-4.

9. Procédé d'isolement de l'antigène CA 125, tel que défini dans la revendication 1, à partir de cellules de carcinome ovarien, comprenant les étapes consistant :

- a) à recueillir un liquide surnageant dépourvu de cellules à partir d'une culture de cellules de carcinome ovarien qui déversent du CA 125 dans le milieu de culture;
- b) à acidifier le liquide surnageant avec de l'acide perchlorique pour précipiter la protéine;
- c) à éliminer la protéine précipitée et à neutraliser la fraction soluble dans l'acide;
- d) à soumettre la fraction soluble dans l'acide neutralisée à une chromatographie d'exclusion de taille moléculaire sur résine Sepharose CL-4B et à récupérer dans la colonne la fraction (volume vide) contenant l'activité de CA 125;
- e) à traiter la fraction contenant l'activité de CA 125 par de l'urée à 6M environ;
- f) à soumettre la fraction traitée par l'urée à une chromatographie d'exclusion de taille moléculaire sur Sepharose CL-6B dans une urée 6M tamponnée et environ 1% de SDS, et à récupérer la fraction éluée contenant l'activité de CA 125;
- g) à éliminer l'urée de la fraction récupérée;
- h) à envoyer la fraction dans une colonne d'immunoaffinité contenant de l'anticorps OC-125 joint à du Sepharose-protéine A par l'intermédiaire de diméthylpimélimidate;
- i) à éluer le CA 125 à partir de la colonne d'immunoaffinité avec de la diéthylamine.

10. Immunoabsorbant pour l'adsorption spécifique d'anticorps réagissant avec CA 125, comprenant une espèce immunoréactive isolée d'antigène CA 125 selon la revendication 1.

11. Composition immunogène pour l'immunisation d'un animal contre l'antigène CA 125, comprenant de l'antigène CA 125 isolé selon la revendication 1 dans un véhicule acceptable physiologiquement.

5 12. Composition immunogène selon la revendication 11, dans laquelle l'antigène CA 125 isolé est une espèce immunoréactive de CA 125 ayant un poids moléculaire d'environ 200 kd.

13. Utilisation de l'immunogène selon la revendication 11 pour la production d'anticorps contre CA 125.

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